HYDROGEN EXCHANGE RATE IN $C_{(8)}H$ GROUPS OF PURINE RESIDUES AS A TOOL FOR ESTIMATION OF THEIR pK_a VALUES IN NUCLEIC ACIDS

R. N. MASLOVA, E. A. LESNIK and Ya. M. VARSHAVSKY

Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow 117312, USSR

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1. Introduction

According to the data described in our previous publications [1–3] the reactivity of purine residues in polynucleotides and nucleic acids, and, in particular, the ability of H-atoms in the $C_{(8)}H$ groups to take part in the hydrogen exchange reaction with water depends on the conformation of the macromolecules, which in turn influences the electronic state of purine residues. To estimate the change of electronic state of purine residues by the rate of $^1H \rightarrow {}^3H$ exchange in the groups mentioned, one should know the mechanism of the exchange reaction.

In the paper by Tomasz et al. [4] data are presented concerning the rate of the isotope hydrogen exchange in the $C_{(8)}H$ groups of purine nucleosides and a suggestion is put forward that the exchange proceeds via the ylide mechanism proposed earlier [5] to explain hydrogen mobility in the $C_{(2)}H$ groups of imidazole and its derivatives.

This report is devoted to the study of $^3H \rightarrow {}^1H$ exchange kinetics in the $C_{(8)}H$ groups of some purine derivatives over a wide pH range. We looked for a correlation between the kinetic data and the ylide mechanism of the exchange reaction and for a relationship between the constant of the exchange rate and affinity of the purine residues for protons (pK_a), arising from their electronic state. It is evident that if such a relationship had been found one would be able to estimate the pK_a values of purine residues in nucleic acids depending on their conformation.

2. Materials and methods

Commercially available [8-^{3}H]guanine and [8-^{3}H]-xanthine were used [8-^{3}H]labelled purine, adenine, hypoxanthine and [2-^{3}H]-labelled imidazole and benzimidazole were obtained after hydrogen exchange in $^1H^3HO$ and purified. Upon chromatographic analysis, a single radioactivity spot was observed for each compound coinciding with the UV-absorbing spot. Spectral characteristics of all the purified ^{3}H-compounds corresponded to those described for non-labelled substances.

To measure the rate of $^3H \rightarrow {}^1H$ exchange ^{3}H-containing compounds were incubated for a given time at 80°C at a given pH value. Specific radioactivity was measured after removal of traces of $^1H^3HO$ by repeated lyophilization. The rate constant of the exchange (k^{obs}) was calculated by using a first order equation:

$$k^{obs} = \frac{2.3}{t} \log \frac{SA_t}{SA_0} \qquad (1)$$

where: 't' is incubation time in seconds; SA_t and SA_0 are the specific radioactivities of the compound under study after and prior to the incubation, respectively.

Radioactivity was measured in an SL-40 liquid scintillation spectrometer (Intertechnique, France). Concentrations were calculated from the values of optical density. Spectral characteristics of the com-

pounds studied were read in a recording Hitachi spectrophotometer in the range from 200 to 300 nm before and after incubation.

3. Kinetic analysis

For the isotope hydrogen exchange in a compound 'A' the following relationship is valid:

$$\text{Exchange rate} = k^{obs} \cdot [A] \qquad (2)$$

where [A] is the total concentration of compound 'A'.

For compounds containing up to 3 ionizing groups one of which is able to accept a proton and two others can donate one, the total concentration of the molecules 'A' is expressed as a sum: $[A] = [A^0] + [AH^+] + [A^-] + [A^{2-}] + [A^{\pm}]$. The terms in this summation represent concentrations of uncharged molecules and those of protonated, deprotonated, twice deprotonated and zwitter-ion forms of the compound 'A', respectively.

According to the ylide mechanism [4,5], hydrogen exchange between water and $C_{(8)}H$ groups of purine residues proceeds in the positively charged ions occurring due to protonation of N-atoms neighbouring to the exchangeable CH-group and in the corresponding zwitter-ions. The exchange rate is restricted by a proton elimination from the $C_{(8)}H$ group by OH^- ions of the solvent. Thus if we assume the ylide mechanism to be valid we can write:

$$\text{Exchange rate} = k^{+}[AH^+][OH^-] + k^{\pm}[A^{\pm}][OH^-] \qquad (3)$$

where k^+ and $k^{\pm}$ are the true constants of the exchange rate in AH^+ and $A^{\pm}$ ions, respectively.

By combining the equations (2) and (3), we obtain:

$$k^{obs}[A] = k^{+}[AH^+][OH^-] + k^{\pm}[A^{\pm}][OH^-] \qquad (4)$$

Substituting in the equation (4) the ratios of ionization constants of the compounds under study and $[H^+]$ for $[A]$, $[AH^+]$, $[A^{\pm}]$ and $K_w[H^+]$ for $[OH^-]$, where K_w is ion product of water, we obtain the general expression for the value of k^{obs}:

$$k^{obs} = \frac{k^{+}\dfrac{K_w}{K_{a_1}} + k^{\pm}K_{zw}\cdot\dfrac{K_w}{[H^+]}}{1 + \dfrac{[H^+]}{K_{a_1}} + \dfrac{K_{a_2}}{[H^+]} + \dfrac{K_{a_2}K_{a_3}}{[H^+]^2} + K_{zw}} \qquad (5)$$

The analysis of equation (5) shows [4] that to calculate k^+ one should know the values of K_w and K_{a_1} and experimentally determine the value of k^{obs} within the range $K_{a_1} \gg [H^+] \gg K_{a_2}$ where this value is constant. This k^{obs} value will be designated as k_1^{obs}:

$$k_1^{obs} = k^{+}\frac{K_w}{K_{a_1}} \qquad (6)$$

To calculate $k^{\pm}$ one has to know the values of K_w, K_{zw} and K_{a_2} and to determine experimentally the value of k^{obs} within the range $K_{a_2} \gg [H^+] \gg K_{a_3}$ where it is constant. This value will be designated as k_2^{obs}:

$$k_2^{obs} = k^{\pm}\frac{K_w \cdot K_{zw}}{K_{a_2}} \qquad (7)$$

4. Results and discussion

Figs. 1 and 2 represent experimental data obtained for pH dependence of k^{obs} of adenine and guanine, respectively, used as examples. The pH dependences of k^{obs} for purine, imidazole and benzimidazole are similar to that of adenine (fig. 1) whereas the curves obtained for hypoxanthine and xanthine resemble that of guanine (fig. 2). It can be seen that in both cases there are pH ranges where k^{obs} does not actually depend on pH. For adenine, such a region is single; for guanine, two regions are revealed. Respective values of k^{obs} (k_1^{obs} and k_2^{obs}) for these regions are given in table 1. In the same table are given the true constants of the exchange rates in protonated and

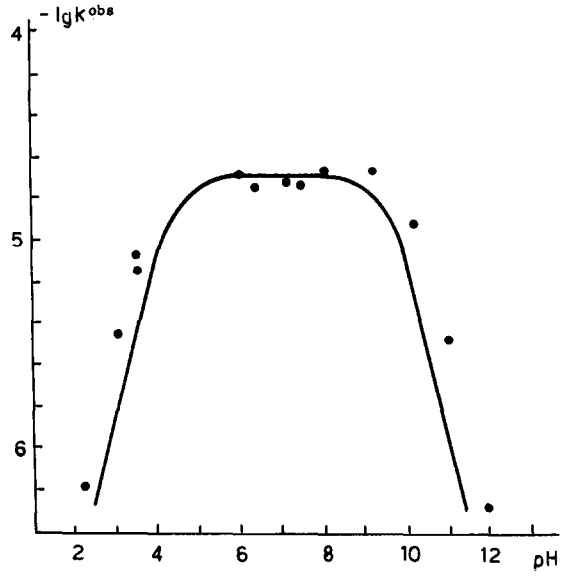


Fig. 1. Comparison of the experimental data and theoretical curve of the observed rate constants of the $^3H \rightarrow {}^1H$ exchange reaction of adenine $C_{(8)}H$ groups. (•) Experimental points of log k^{obs}; (——) theoretical curve of log k^{obs} calculated from eq. (5).

zwitter-ion* forms calculated by using equations (6) and (7).

The theoretical curves in figs. 1 and 2 correspond to the values k^{obs} calculated according to equation (5) with the use of the k_1^{obs} and k_2^{obs} values presented in table 1. In these calculations we have assumed that in the case of adenine (and compounds I–III), a single exchangeable form is represented by protonated molecules whereas in the case of guanine (and compounds V–VII), in addition to protonated molecules, zwitter-ions carrying a positive charge at the $N_{(7)}$ atom are also involved in the exchange. This assumption was based on the distinction mentioned above in the shapes of the k^{obs} versus pH curves for adenine and guanine. Dotted lines (a,b) in fig. 2 correspond to the pH dependence of k^{obs} calculated for the protonated and zwitter-ion guanine molecules,

* Since the data on the K_{zw} values necessary for calculation of $k^{\pm}$ (see eq. (7)) are not available from the literature (except for K_{zw} for guanosine [4]) we calculated the product of $k^{\pm} \cdot K_{zw}$.

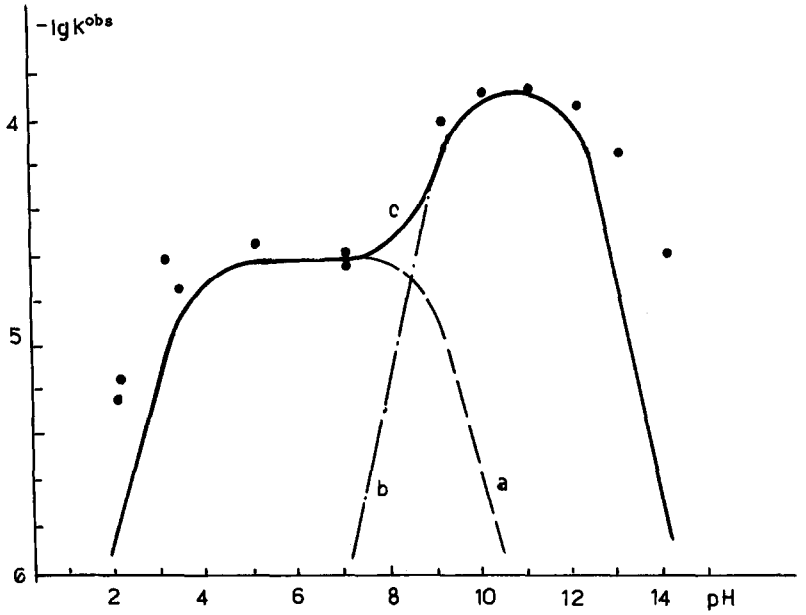


Fig. 2. Comparison of the experimental data and theoretical curve of the observed rate constants of the $^3H \rightarrow {}^1H$ exchange reaction of guanine $C_{(8)}H$ groups. (•) Experimental points of log k^{obs}; (——) theoretical curve (c) of logarithms over all k^{obs} calculated from eq. (5); (- - - -) theoretical curve (a) of log k^{obs} for protonated molecules; (–·–·–) theoretical curve (b) of log k^{obs} zwitter-ions.

Table 1
The constants of ionization (K_a) [8,9] and the rate constants of the $^3H \rightarrow {}^1H$ exchange of the compounds studied

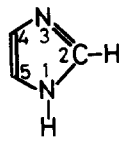
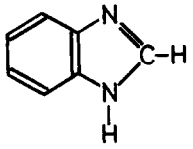
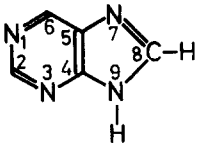
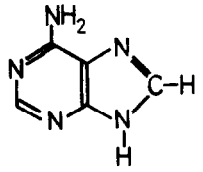
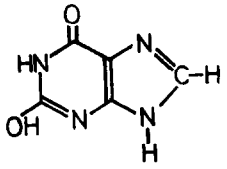
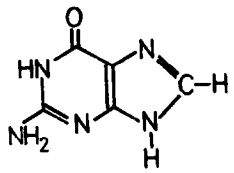
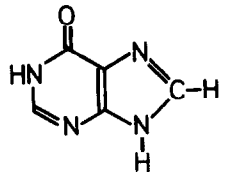
Compound	pK_a			Interval pH in which k^{obs} = const.	k^{obs}		k^+	$k^{\pm}.K_{zw}$
	pK_{a_1}	pK_{a_2}	pK_{a_3}		k_1^{obs}	k_2^{obs}	(sec^{-1})	(sec^{-1})
Imidazole (I)	7.0							
		13		8–11	$5.0 \cdot 10^{-4}$	–	$1.3 \cdot 10^2$	–
Benzimidazole (II)	5.5							
		12.3		6–10	$5.2 \cdot 10^{-4}$	–	$4.1 \cdot 10^3$	–
Purine (III)	2.4							
		8.9		3–7	$1.3 \cdot 10^{-5}$	–	$1.3 \cdot 10^5$	–
Adenine (IV)	4.2							
		9.8		6–9	$2.1 \cdot 10^{-5}$	–	$3.3 \cdot 10^3$	–
Hypoxanthine (V)	1.98							
		8.99		3–7	$1.2 \cdot 10^{-5}$		$3.3 \cdot 10^5$	
				9–11		$5.0 \cdot 10^{-5}$		$1.4 \cdot 10^{-1}$
			12.1					

Table (continued)

Guanine (VI)	3.3					
		9.2	4–7	$2.5\cdot10^{-5}$	$3.1\cdot10^{4}$	
			10–11	$1.4\cdot10^{-4}$		$2.2\cdot10^{-1}$
		12.3				
Xanthine (VII)	0.8					
		7.44	2–5	$3.7\cdot10^{-6}$	$1.5\cdot10^{6}$	
			8–10	$5.0\cdot10^{-5}$		4.7
		11.2				

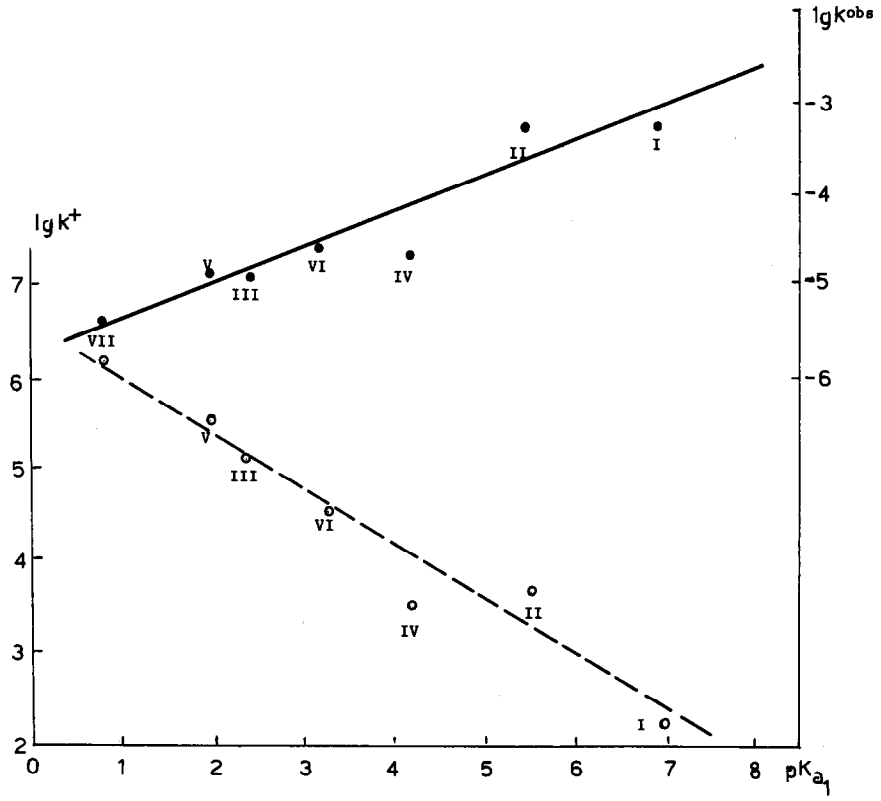


Fig. 3. pK_{a_1}-dependence of logarithms of the rate constants of the $^3H \rightarrow {}^1H$ exchange between water and CH-groups of the compounds I–VII. (•) Experimental points of $\log k^{obs}$; (○) the values of $\log k^{+}$ calculated from eq. (6).

respectively. It can be seen that the experimental data both for adenine (fig. 1) and guanine (fig. 2) coincide rather well with the curves calculated on the basis of the ylide mechanism. Similar coincidence of experimental and calculated data was observed also for all the compounds studied in this work. Some shifts of the experimental points towards lower pH values observed in the protonation region for all the compounds may be accounted for by the fact that the calculations were performed by using the ionisation constant determined at 20°C whereas experimental data were obtained at 80°C.*

Fig. 3 shows ratios between k^+ values for all the compounds under study listed in table 1 and respective protonation constants (K_{a_1}) on logarithmic scales. It can be seen that the lowest k^+ value is inherent to the protonated form of the compound with the greatest proton affinity, viz. imidazole. In the series imidazole, benzimidazole, purine and oxypurine (i.e. on decreasing order of proton affinity) the k^+ value increases. If the ylide mechanism is assumed this fact shows that the weaker the ability of molecules to bind a proton the easier is the elimination of a proton from the $C_{(8)}H$ groups of the protonated molecules by OH^-ions. Linear dependence between $\log k^+$ and pK_{a_1} shown as dotted curve in fig. 3, favours the assumption on the general mechanism of the exchange reaction for all the compounds under study. The slope of the straight line (about 0.6) may serve as a quantitative characteristic of this dependance; a 10-fold decrease of K_{a_1} corresponds to a 4-fold increase of k^+.

It should be emphasized that the above-mentioned linear dependance corresponds to the k^+ value which was not measured directly but calculated from equation (6) using the experimentally obtained k_1^{obs} values, and known values of K_{a_1}. The solid line in fig. 3 shows that the values of experimentally measured k_1^{obs} in contrast to k^+, decrease upon a decrease in pK_{a_1} (that is upon decreased proton affinity). The relationship k_1^{obs} and pK_{a_1} is of practical interest for the compounds whose pK_{a_1} values are not known. We mean, in particular, purine residues in polynucleotides and nucleic acids with ordered secondary structure since in these cases pK_{a_1} values are known to depend on the conformation of a respective macromolecule.

Measurements of the $^3H \rightarrow {}^1H$ exchange rate at different temperatures have shown that the values of activation energy for all the compounds under study is 21±1 kcal/mole. This makes it possible to suggest that a decrease in the exchange rate upon a fall in temperature should not be accompanied by any changes in the slopes of the curves presented in fig. 3. This means that the $\Delta \log k_1^{obs}/\Delta\, pK_{a_1}$ value found at 80°C (0.39) should be maintained for lower temperatures more typical of experiments with biopolymers. Measuring in the experiment (within the pH range $K_{a_1} \gg [H^+] \gg K_{a_2}$) a change of the k_1^{obs} value due to the change in the macromolecular conformation and using the ratio $\Delta \log k_1^{obs}/\Delta\, pK_{a_1} = 0.39$ one can estimate a change of pK_{a_1}.

Data published in our previous papers [1–3] have shown that the ordering of the secondary structure of polynucleotides and appearance of stacking interaction between the bases is accompanied by a retardation of hydrogen exchange. In view of the facts mentioned these data may be interpreted as indicating a decrease of proton affinity of purine residues after the ordering of the secondary structure.

In this connection it was interesting to evaluate the changes in pK_{a_1} of purine nucleotides and their residues within the double-stranded DNA molecule by using the values of ratio constants of hydrogen exchange in $C_{(8)}H$ groups. Previously it has been shown [6,7] that for DNA incubated in 1 × SSC in the range of 35–70°C an independance of the coefficient of exchange retardation ($K_r = k_1^{obs}$ (of NMP mixture): k_1^{obs} (of DNA)) on the temperature is observed. The K_r value for DNA with 50% (G + C) content was found to be 2.4. Therefore, for this DNA

$$\Delta \log k_1^{obs} = \log k_1^{obs} \text{ (of NMP mixture)} - \log k_1^{obs} \text{ (of DNA)} = 0.38.$$

By using the above relation we find that

$$\Delta pK_{a_1} = pK_{a_1} \text{ (of NMP mixture)} - pK_{a_1} \text{ (of DNA)} = 0.38/0.39.$$

* It should be noted that the experimental values of k^{obs} at pH > 12 (figs. 1 and 2) are rather approximate since we failed to carry out accurate measurements in such strongly alkaline solutions.

Thus, pK_{a_1} of purine residues within double-stranded DNA according to this calculation should be about one unit lower than pK_{a_1} of the respective nucleotides.

Direct measurements of pK_{a_1} for the purine nucleotide residues within the double-stranded DNA cannot be performed since their protonation results in DNA denaturation. One may only notice that the value of $\Delta pK_{a_1} = 0.98$ found by us (for purine residues of DNA (in 1 × SSC)) agrees rather well with the values of 0.8 and 1.5 which were obtained [10] upon measuring the degree of protonation of cytidylic residues of double-stranded DNA in 0.02 M KCl and 0.2 M KCl, respectively.

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